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Comparison of flow cytometric and epifluorescent counting methods for marine heterotrophic bacteria

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Two direct heterotrophic bacterioplankton counting methods, epifluorescence microscopy (EM) and flow cytometry (FCM) were compared using samples collected in two geographically different oceanic regions, the Adriatic Sea and the English Channel. A statistically significant correlation was found between results obtained by these two methods for samples collected in the Adriatic Sea ($r = 0.61$, $n = 919$, $P < 0.001$) and in the English Channel ($r = 0.64$, $n = 33$, $P < 0.001$). Samples from the Adriatic Sea showed on average 1.16 times higher values obtained by flow cytometry than values estimated by epifluorescence microscopy, while samples from the English Channel showed on average a 0.74 ratio between flow cytometry and epifluorescence microscopy counts. The overall coefficient of variation for epifluorescence microscopy data for samples from the Adriatic Sea and the English Channel was 15.91% and 12.89%, respectively. The flow cytometry method had lower overall coefficient of variation value; for samples collected in the Adriatic Sea it was 3.64%, while for samples collected in the English Channel it was 1.88%.

Key words: Epifluorescence microscopy, flow cytometry, heterotrophic bacterial abundance

INTRODUCTION

During the past two decades total direct counting methods for enumerating heterotrophic bacteria that use fluorochrome stains and epifluorecence microscopy have become very popular and widely used. Until recently, most determinations of heterotrophic bacterial abundance were usually done by epifluorescence microscopy of DAPI (4'-6-diamidino-2-phenylindole) or Acridine Orange stained samples (HOBBIE *et al*., 1977; PORTER & FEIG, 1980). For optimum accuracy, it is recommended to count about 400 cells on multiple filters (FRY, 1990), making this method both time consuming and subjective. Recently, a new generation of methods, including flow cytometry (BURKILL, 1987; OLSON *et al*., 1990) and image cytometry (SIERACKI *et al.*, 1995) became available. These methods significantly reduce the time employed in each of these determinations (multiparameter analysis of individual cells); they increase the level of resolution and provide new insights into the structure and func-

tioning of plankton communities that simply can not be obtained with conventional epifluorescence microscopy (LI *et al*., 1995; MARIE *et al.*, 1996, 1997). Flow cytometry has been routinely used for the analysis of marine samples and is now commonly accepted as a reference technique in oceanography and for the analysis of the heterotrophic bacterial community (MONGER & LANDRY, 1993). Microscopic enumeration is less accurate than flow cytometric enumeration due to several factors including a low number of cells that are counted (~ 400) and the non-homogeneous distribution of cells on the membrane filter. Further, the presence of organic and mineral particles and the small sizes of most marine heterotrophic bacteria may result in lower bacterial discrimination when cells are counted by microscopy (LEBARON *et al*., 1993). The advantages of flow cytometry are the rapid and automated analyses that separate heterotrophic bacteria from other particles on the basis of light scatter (size) and pigment content and that gives greater precision than epifluorescence counting (SIERACKI *et al.*, 1995; MONGER & LANDRY, 1993; JOACHIMSTHAL *et al*., 2003; CHISHOLM *et al*., 1988).

The objective of this study was to compare two direct counting methods for heterotrophic bacterioplankton and the field samples from different oceanographic regions - the Adriatic Sea and the English Channel. The accuracy of epifluorescence microscopy (EM) was assessed against direct counts made by flow cytometry (FCM).

MATERIAL AND METHODS

Samples were collected in two geographically different areas; the Adriatic Sea, as part of the Mediterranean Sea (Fig. 1) and the English Channel, as part of the Atlantic Ocean (50°15'N; 4°15'W, offshore station 6 km off Plymouth, and four shore stations from Plymouth Sound UK). From the Adriatic Sea a total of 919 samples, spanning offshore and shore areas, were col-

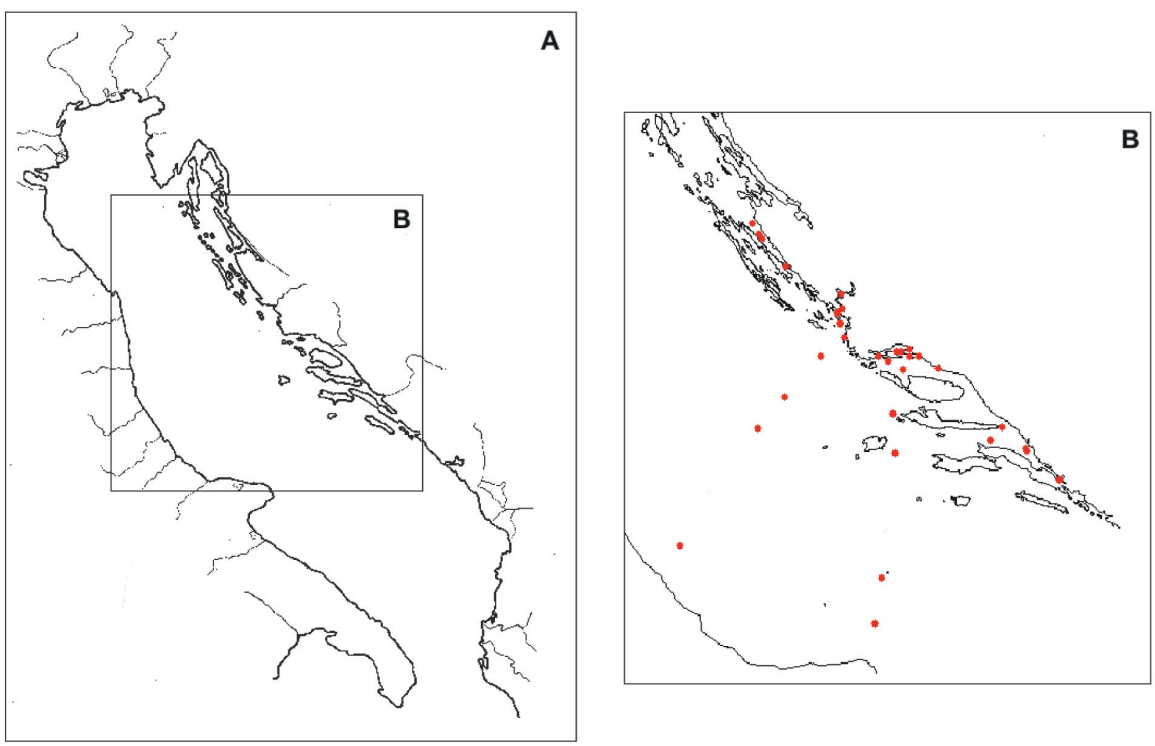

Fig.1. (A) The Adriatic Sea (B) Locations of the investigated sites in the Middle Adriatic Sea

lected on a monthly basis from 29 sites during 2005.

From the English Channel (N = 132) samples were collected at weekly to monthly intervals during winter 2006 from one offshore and four shore sites. In addition, for the purpose of testing repeatability and counting precision, four replicates were separately made by both direct counting methods for samples from both the Adriatic Sea and the English Channel.

Seawater samples from the Adriatic Sea sites and the offshore site in the English Channel were collected by Niskin bottles through vertical profiles. At the four shore sites in the English Channel samples were collected manually from the surface. Samples were fixed with formaldehyde (2% final concentration), kept in the dark at 4 ^{0}C and analyzed within two weeks.

For epifluorescence microscopy (EM) preserved samples were stained with 4'-6-diamidino-2-phenylindole (DAPI) (1 μg mL^{-1} final concentration) for 5 minutes and were filtered through 0.2 μm pore diameter black polycarbonate filters (Millipore, Ireland). Filters were then mounted on microscope slides and stored at 4 ^{0}C where they were kept until observation with an Olympus microscope under UV light (PORTER & FEIG, 1980) at a magnification of 1000. From 100 to 400 heterotrophic bacteria were counted per sample, depending on concentration.

For flow cytometry analysis (FCM), fixed samples were stained with SYBR green I (Molecular probes Inc.) (MARIE *at al*., 1997; LEBARON *et al*., 1998). Samples from the Adriatic Sea were analyzed on a Beckman Coulter EPICS XL-MCL with a high flow rate from 1 to 1.2 μL/sec. Fluorescent beads were added (Level-II Epics Division of Coulter Corporation Hialeah, Florida) for calibration of fluorescence intensity. Samples from the English Channel were analyzed on a flow cytometer FACSort. Beckman Coulter flow set beads at known concentration were used to calibrate the flow rate. Bacterial abundance was determined in scatter plots of particle side scatter versus SYBR green I fluorescence related to cellular nucleid acid content to discriminate heterotrophic bacteria from other particles.

Correlations were determined using the Spearman rank correlation index (ZAR, 1984). The statistics software STATISTICA v. 6.0. was used in all analyses.

RESULTS AND DISCUSSION

A statistically significant correlation was found between heterotrophic bacterial counts measured by microscopy and flow cytometry for samples collected in the Adriatic Sea ($r = 0.61$, $n = 919$, $P < 0.001$) and in the English Channel ($r = 0.64$, $n = 33$, $P < 0.001$) (Fig. 2). Similar significant correlations ($r^2 > 0.8$) were also found in the northwestern Mediterranean Sea (LEBARON *et al*., 1993, 1998; GASOL *et al*., 1999). A statistically significant correlation between the two methods was also observed on a monthly basis. The lowest, though still significant correlation was found during September and November (Table 1). A possible explanation for these low correlations could be the high content of *Prochloroccocus* recorded during September and November (ŠANTIĆ 2005, personal comunication). High concentrations of *Prochloroccocus* can cause a significant overestimation of heterotrophic bacteria counts (SIERACKI *et al*., 1995) as it can not be distinguished from heterotrophic bacteria by epifluorescence microscopy although could be distinguished by flow cytometry (CHISHOLM *et al*., 1988).

Table 1. Correlations in bacterial abundances obtained by epifluorescence microscopy and flow cytometry methods at the stations in the Adriatic Sea during 2005

Month	N	Spearman rank correlation (r)	P value
January	74	0.74	<0.01
February	47	0.79	<0.01
March	26	0.81	<0.01
April	22	0.79	<0.01
May	117	0.52	<0,01
June	92	0.80	<0.01
July	92	0.49	<0,01
August	135	0.75	<0.01
September	128	0.43	<0.01
October	26	0.77	<0.01
November	68	0.39	<0.01
December	92	0.64	<0.01

Counts of heterotrophic bacteria obtained by flow cytometry and microscopy were more similar in the Adriatic Sea than in the English Channel. Analysis of variance did not show a significant difference between those counts determined by FCM and EM in the Adriatic Sea ($P > 0.05$), whereas data from the English Channel showed significant difference ($P < 0.05$).

The ratio between counts by FCM and EM for Adriatic stations averaged 1.16. Total heterotrophic bacterial cell numbers varied from 0.05×10^6 to 3.92×10^6 mL^{-1} using the microscopy method and from 0.09×10^6 to 3.67×10^6 cells mL^{-1} using the flow cytometry method.

The ratio slightly above 1 in flow cytometry versus epifluorescence microscopy measurements was also found in earlier studies (MONGER & LANDRY, 1993; LEBARON *et al*., 1998; GASOL *et al*., 1999; JOCHEM, 2001) and was explained by underestimation of bacterial abundance by microscopy since 2 to 6% of small heterotrophic bacterial cells were found to pass through 0.2 µm membrane filters (GASOL & MORÀN, 1999).

The comparison of data from counts made by epifluorescence microscopy and flow cytometry in samples from the English Channel showed higher values for heterotrophic bacteria counts made by epifluorescence microscopy, where the average ratio between bacterial counts obtained by FCM and microscopy

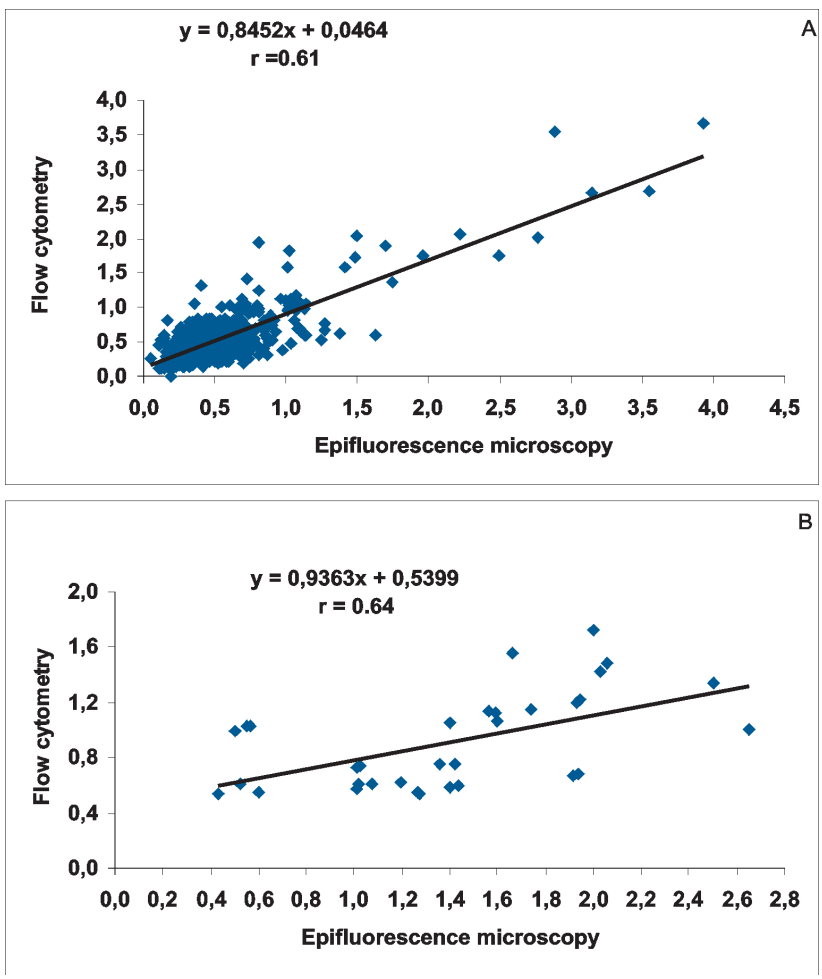

Fig. 2. Relationship between total bacteria numbers (10 ^{6} cell mL^{-1}) estimated by epifluorescence microscopy and flow cytometry in the Adriatic Sea (A) and the English Channel (B)

was 0.74. Total heterotrophic bacteria counts from sites in the English Channel estimated by microscopy varied from 0.43 x 10^6 to 2.65 x 10^6 cells mL^{-1} and by FCM from 0.62 x 10^6 to 1.73 x 10^6 cells mL^{-1}. The most likely reason is that between January and March the English Channel has a lot of suspended material in the water which might be stained by DAPI and get counted as heterotrophic bacteria. Due to the gating of data the flow cytometry method will only take into account cells in a particular region of interest while the microscopy method results in counts that also include algae and protozoa (JOACHIMSTHAL *et al.*, 2003). Some researchers presented evidence that a large fraction of what were then being counted as heterotrophic bacteria in DAPI stained samples for microscopy were in fact particles without genome: dead cells, ghosts or large viruses and cell fragments (GASOL *et al.*, 1999; ZWEIFEL & HAGSTRÖM, 1995).

The ratio between heterotrophic bacteria counts obtained by flow cytometry and microscopy (FCM: EM ratio) and total heterotrophic bacteria numbers showed statistically significant correlations both in the English Channel ($r = 0.65$, $n = 33$, $P < 0.05$) and in the Adriatic Sea ($r = 0.41$, $n = 147$, $P < 0.05$) (Fig. 3). These results suggested that an increase in total heterotrophic bacteria count is accompanied by an increase in FCM: EM ratio.

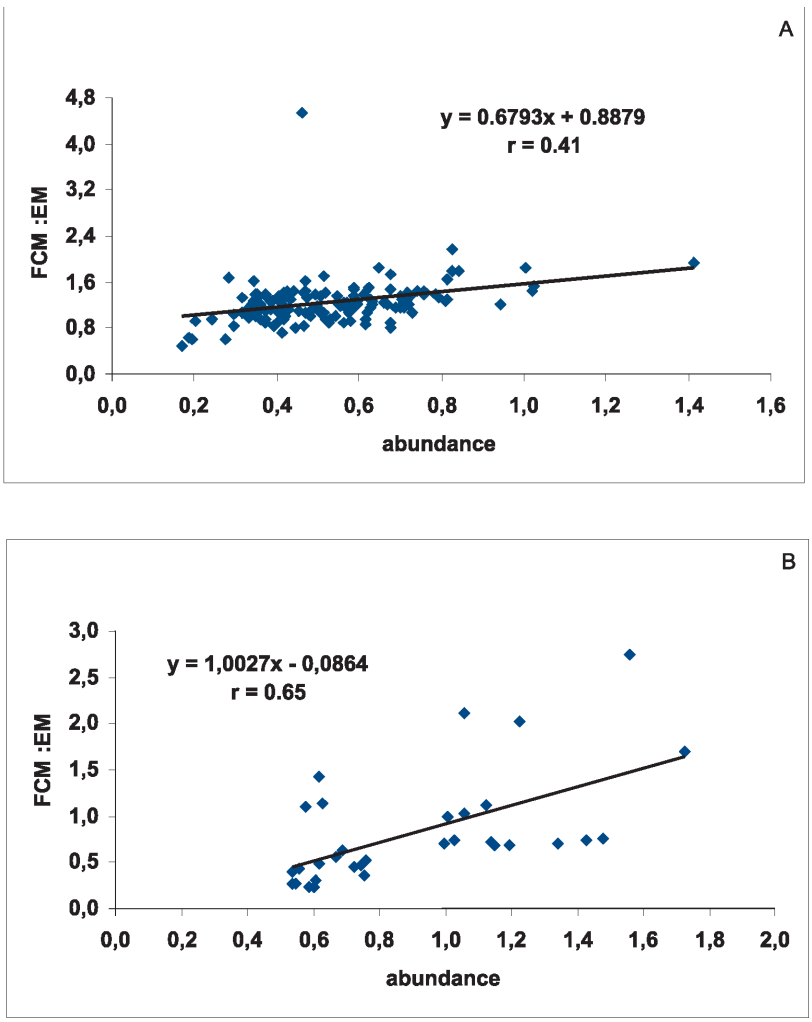

Fig. 3. Relationship between total bacteria numbers (10 ^{6} cell mL^{-1}) estimated by flow cytometry and FCM: EM ratio in the Adriatic Sea (A) and the English Channel (B)

Replicate experiments in both investigated areas showed that coefficients of variation were lower for heterotrophic bacteria counts estimated by FCM than by microscopy (Fig. 4).

The overall coefficient of variation for epifluorescence microscopy data for samples from the Adriatic Sea was 15.91% (ranging from 4.48% to 28.11%), while for samples from the English Channel it was 12.89% (ranging from 3.29% to 21.76%). On the other hand the overall coefficient of variation for flow cytometry data for samples collected from the Adriatic Sea was 3.64% (ranging from 0.93 to 10.06%), while from the English Channel it was 1.88% (ranging from 0.88 to 3.44%). A similar coefficient of variation for flow cytometry (average 1.5%) was presented by other researchers in the area of Hawaii (MONGER & LANDRY, 1993).

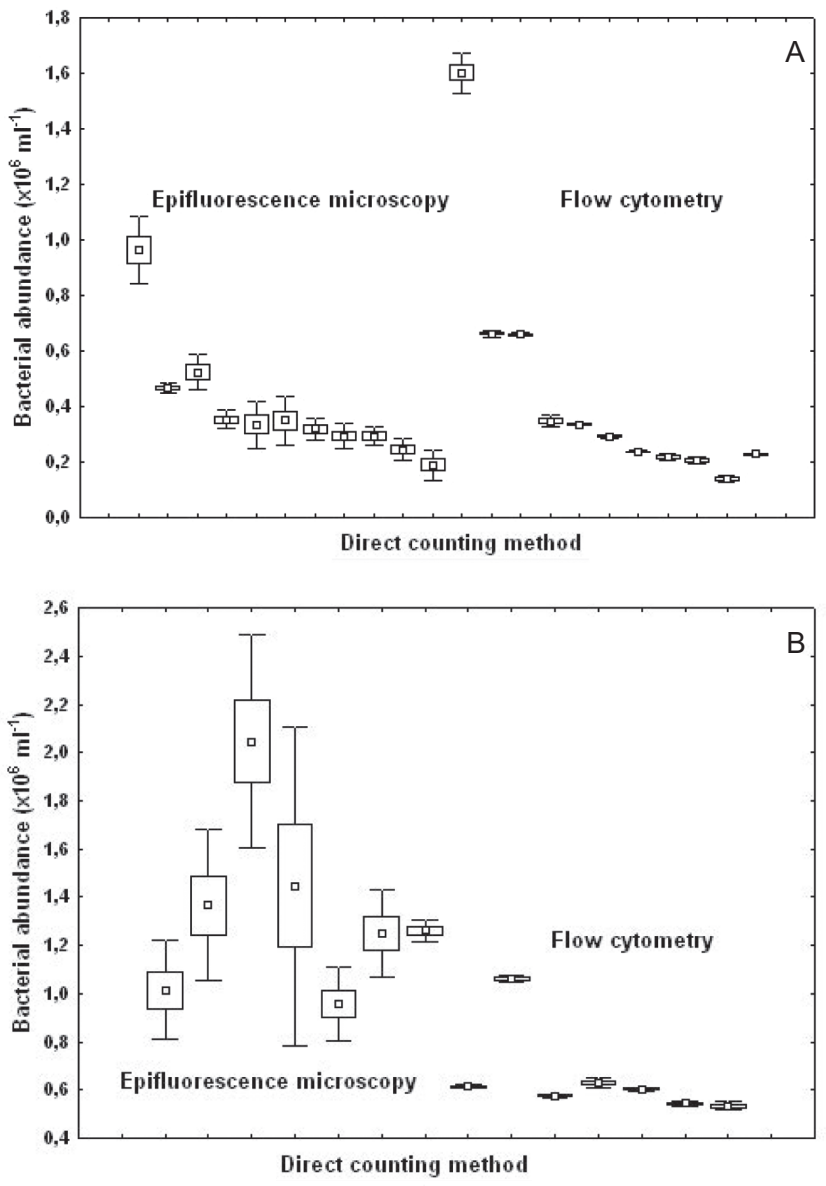

Fig. 4. Box-Whisker (mean; 50% conf. int.; std. dev) plot of bacterial abundance obtained by epifluorescence microscopy and flow cytometry from the Adriatic Sea (A) and the English Channel (B)

In conclusion, the results reported here showed a significant relationship between epifluorescence microscopy and flow cytometry and also showed that coefficients of variation were considerable lower for heterotrophic bacteria counts estimated by flow cytometry. This paper supports previous recommendations of flow cytometry as a rapid, automated and accurate counting method.

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Usporedba metoda protočne citometrije i fluorescentne mikroskopije za brojenje morskih heterotrofnih bakterija

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SAŽETAK

Dvije direktne metode za brojanje heterotrofnog bakterioplanktona, epifluorescentna mikroskopija (EM) i protočna citometrija (FCM), uspoređene su koristeći uzorke sakupljene s dva zemljopisno različita morska područja, Jadranskog mora i Engleskog kanala. Između dvije direktne metode brojanja u uzorcima iz Jadranskog mora ($r = 0.61$, $n = 919$, $P < 0.001$) i Engleskog kanala ($r = 0.64$, $n = 33$, $P < 0.001$) pronađena je statistički značajna korelacija. U Jadranskom je moru metoda protočne citometrije davala u prosjeku neznatno niže rezultate u odnosu na metodu epifluorescentne mikroskopije, dok je u Engleskom kanalu broj heterotrofnih bakterija dobiven metodom epifluorescentne mikroskopije bio u prosjeku nešto veći u odnosu na protočnu citometriju. Međutim, srednji koeficijent varijacije rezultata dobivenih epifluorescentnim mikroskopom za područje Jadrana iznosio je 15.91%, za područje Engleskog kanala 12.89%, dok je protočna citometrija rezultirala nižim koeficijentima varijacije te je za uzorke iz Jadrana iznosila 3.64%, a za uzorke sakupljene u Engleskom kanalu svega 1.88%. Navedeni rezultati predstavljaju doprinos sagledavanju metode protočne citometrije kao brže, sigurnije i preciznije za brojenje bakterioplanktona u morskom okolišu.

Ključne riječi: epifluorescentna mikroskopija, protočna citometrija, gustoća heterotrofnih bakterija